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Mechanisms of calcium oxalate crystal attachment to injured renal collecting duct cells

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Mechanisms of calcium oxalate crystal attachment to injured renal collecting duct cells.

Background. Renal cell or tissue injury results in a loss of membrane lipid asymmetry and/or loss of cell polarity, and both events lead to changes on the surface of the cell membranes that enhance crystal attachment. We have proposed two distinct mechanisms of crystal attachment following membrane changes induced by various modes of injury.

Methods. Annexin V was used to determine whether phosphatidylserine (PS) exposure on the cell membrane surface plays a role in calcium oxalate monohydrate (COM) crystal attachment to cells that have lost their polarity as well as to cells that have lost their lipid asymmetry. We utilized two different experimental models of injury to renal epithelial cells in culture. The first model used calcium ionophore A23187 to induce a loss of lipid asymmetry, and the second model used EGTA to break down tight junctions and lose cell polarity.

Results. Inner medullary collecting duct cells that have lost lipid asymmetry demonstrated an increase in the number of cells that bound annexin V. However, when cells lost their polarity, they did not bind annexin V. In addition, the attachment of crystals to cells following a loss of cell polarity was not inhibited by annexin V.

Conclusions. This study indicates that both individual cell injury (loss of lipid asymmetry) and generalized cell monolayer injury (loss of cell polarity) result in the presentation of different cell surfaces and that both forms of injury result in an increased affinity for crystal attachment. Both mechanisms could be important independently or collectively in the retention of microcrystals to renal collecting duct cells in urolithiasis.

The retention of microcrystals by the kidney urothelium is thought to be a necessary and critical event in the growth of renal calculi. There is evidence that renal cell or tissue injury results in structural, compositional, and physiological changes in the cell plasma membrane that enhance crystal attachment. It has been shown in rat models and in humans that crystal retention intimately involves the urothelial cell membrane [1–4]. The interaction between crystals and cell membranes in urolithiasis appears to be mediated by specific interactions between stone crystal surface structures and molecular arrays on the surfaces of cell membranes [5–7]. The exact nature of this crystal–membrane interaction is still unknown. The main difficulty in elucidating the molecular mechanisms of the crystal–membrane interaction is the complex and dynamic nature of the cell membrane.

Crystal retention by urinary epithelium is thought to be enhanced by prior injury. Both Gill, Ruggiero, and Straus [8] and Khan et al [2, 9] have shown that calcium oxalate attachment to the urothelium of the rat bladder increases following injury by either Triton X-100 or HCl when compared with uninjured urothelium. The mechanisms involved in crystal attachment to renal epithelial cells and injury to renal epithelial cells have been studied extensively by us using inner medullary collecting duct (IMCD) cells and by others using various epithelial cells, including Madin-Darby canine kidney (MDCK), pig kidney proximal tubular epithelial (LLC-PK_1), and the African green monkey epithelial cell line (BSC-1) [10–22]. It has been shown in several cell types that high oxalate levels and/or calcium oxalate crystals can produce cell injury and altered cell physiology that can have an impact on crystal attachment [23–30]. It has also been shown that oxalate induces the exposure of phosphatidylserine (PS) on the surface of IMCD cells and was temporal to the attachment of COM crystals [31].

We suggest that the molecule or molecules involved in crystal attachment must satisfy the specific criteria detailed in this article. First, the cell surface crystal attachment molecule(s) is not present on the surfaces of normal healthy urothelial cells, but is present on the surfaces of cells with abnormal physiology related to the challenges of injurious urinary conditions. Second, a crystal attachment molecule(s) on cell surfaces must

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be available at times when crystals attach and stones begin to form. Third, the crystal attachment molecule(s) must be an integral part of the plasma membrane and must be present on the outer leaflet of the membrane to attach crystals firmly. Fourth, there must be a reasonable expectation that the crystal attachment molecule(s) would be present on the cell surface in sufficient numbers to attach and retain crystals under defined urinary conditions. Finally, there should be a defendable hypothesis of how in vivo stone-forming urinary conditions would induce altered membrane composition and/or structure supportive of crystal attachment.

A study on the influence of various phospholipids in the IMCD cell membrane on crystal attachment [10] and a study in which IMCD cells were injured with calcium ionophore A23187 [12] have suggested that PS exposure on the cell surface may be responsible for increased crystal attachment. PS is normally sequestered to the inner leaflet of the plasma membrane and becomes exposed on the cell surface following cell injury or apoptosis. Annexin V, a protein that specifically binds to PS [32], inhibited COM attachment to IMCD cells treated with calcium ionophore A23187 providing convincing evidence of the role of PS in crystal attachment [12].

When kidney cells in culture were treated with ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,N′-tetraacetic acid (EGTA) to break tight junctions [19], which resulted in the rearrangement of basolateral components to the luminal cell surface, the attachment of preformed calcium oxalate monohydrate (COM) crystals to the cells was dramatically increased. It has also been shown that when strips of cells are scraped from an MDCK cell monolayer in culture, increased COM attachment occurs to the underlying substrate and to cells that have lost polarity on the periphery of the scraped area [21].

The increase in COM attachment when cells in culture lose their polarity was speculated to be due to either exposure of basement membrane components or the rearrangement of basolateral membrane components to the luminal cell surface that facilitate crystal attachment [19]. Membrane rearrangement was confirmed using a monoclonal antibody to a basolateral membrane molecule that was not present on the apical surface of the polarized cells. Unlike the loss of lipid asymmetry where the number of types of newly exposed lipids is few, the multitude of basolateral, basement membrane, and junctional components makes it extremely difficult to speculate on the specific molecules involved in enhanced crystal attachment to cells as a result of loss of cell polarity.

In the present study, using fluorescently labeled annexin V, we determined whether PS exposure on the cell membrane surface plays a role in COM crystal attachment to cells that have lost their polarity paralleling our observations with cells that have lost their lipid asymmetry. We have used two distinctly different models of cell injury that impact crystal attachment to IMCD cell cultures, that is, the loss of cell polarity and the loss of membrane lipid asymmetry.

METHODS

Cell culture

Continuous rat IMCD cells (cIMCD), a generous gift of Dr. John Schwartz [33], were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham base (SFFD) and were supplemented with insulin, hydrocortisone, transferrin, tri-iodo-L-thyronine (T3), selenium, and penicillin/streptomycin (all tissue culture components were purchased from Sigma Chemicals, St. Louis, MO, USA). Cells were plated on round glass cover slips (12 mm diameter), placed in 35 mm diameter tissue culture dishes, at 5×10^4 cells/cover slip, and grown to confluence over three to four days.

Calcium ionophore and EGTA treatment

Inner medullary collecting duct cells were either treated with the calcium ionophore A23187 (Sigma Chemicals) at a final concentration of 5 μmol/L in media for 30 minutes at 37°C or with EGTA (3 mmol/L final concentration) and were incubated for 15 minutes at 37°C. The cells were then washed with either artificial urine [34] or media as described later in this article in the assay procedures.

Calcium oxalate crystal preparation

Radiolabeled COM crystals were prepared as described previously [17]. Ten mmol/L $CaCl_2$ was added to an equal volume of 10 mmol/L sodium oxalate (2 μCi, ^{14}C-oxalic acid) in artificial urine buffer, pH 6.0 [34]. After standing at room temperature for 10 minutes, COM crystals were centrifuged at $1000 \times g$ for 20 minutes. The supernatant was decanted, and the COM crystals were dried overnight under vacuum. Crystal purity was determined by high-resolution x-ray powder diffraction. Crystal morphology was examined by light microscopy and was found to be primarily spherulitic, about 1 μm in diameter.

Addition of exogenous phosphatidylserine to IMCD membranes

Phosphatidylserine was purchased from Avanti Polar Lipids (Alabaster, AL, USA). PS unilamellar liposomes were prepared in phosphate-buffered saline (PBS) by sonication at 4°C for two to three minutes, using a Heat Systems-Ultrasonics Cell Disrupter microtip sonicator [10]. The liposomes were centrifuged at $10{,}000 \times g$ for five minutes to pellet any particulate matter. The liposomes were diluted to a final concentration of 0.4 mg/mL in media without calcium. IMCD cells were washed twice with phosphate buffer and then incubated with 2 mL of

a PS liposome suspension for 30 minutes at 37°C. The cell cultures were then washed twice with artificial urine before crystal attachment experiments were preformed.

Crystal attachment assay

Inner medullary collecting duct cells grown to confluence on glass cover slips were examined under the microscope to insure the cover slips were completely covered with cells. The cells were then washed twice with artificial urine and incubated with 2 mL/35 mm culture dish of a well-dispersed 0.5 mg/mL radioactive crystal suspension for 20 minutes at 37°C. Nonattached crystals were washed free by adhering the cover slips to a microscope slide with a dot of Vaseline and dipping the slide for one minute into a slowly stirred beaker of artificial urine kept at room temperature. The cover slips were placed directly into a 5 mL scintillation vial, covered with 0.5 mL of 6 N HCl, and filled with 3.5 mL of scintillation cocktail (Econosafe; RPI Inc., Mt. Prospect, IL, USA), and the radioactivity was measured using a Packard Tri-Carb scintillation counter (Packard Inst., Meriden, CT, USA).

Fluorescein isothiocyanate-annexin V binding

Fluorescein isothiocyanate (FITC)-annexin V (Annexin V-Fluos) was purchased from Boehringer-Mannheim (Indianapolis, IN, USA) and was used at a working concentration of 1 μmol/L in annexin binding buffer, pH 7.4, containing 10 mmol/L HEPES, 140 mmol/L NaCl, and 5 mmol/L $CaCl_2$. IMCD cells were grown on glass cover slips and rinsed in PBS, and 15 μL of FITC-annexin V solution was applied and incubated for 15 minutes at 37°C. The cells were washed free of excess annexin V with annexin V binding buffer. FITC-annexin V provides for a visual analysis of PS exposure on the individual cells in the cultures. The cells were observed with a Nikon Optiphot-2 microscope using filter B1E (EX 470-490 nm, EM 520-560 nm). Photos were taken with a Nikon U-III photomicrographic unit.

Annexin V inhibition of crystal attachment

Annexin V (Sigma Chemicals) was incubated with IMCD cells before crystal attachment. Confluent cultures of IMCD cells after various experimental treatments were washed two times with 2 mL of annexin-binding buffer. Then 30 μL of 5 μmol/L annexin V, in annexin binding buffer, was added to each cover slip and incubated for 15 minutes at 37°C. The cells were washed free of excess annexin V, and the COM attachment assay was conducted as described previously in this article.

RESULTS

We have visualized PS exposure on IMCD cells in culture using FITC-labeled annexin V, a protein that binds specifically to PS. As a positive control of annexin V binding to PS in membranes, we have visualized the surface binding of annexin V to IMCD cells before (Fig. 1A) and after (Fig. 1B) exposure of the cells to PS containing liposomes [10]. It is clear from Figure 1A, which demonstrates virtually no FITC fluorescence, that under normal conditions, there is very little PS exposed on the cell surface. When exogenous PS was added to the membrane, considerable PS is present on the cell surface, as demonstrated by the increased fluorescence (Fig. 1B).

As can be seen in Figure 2A, treatment of IMCD cells with EGTA to break tight junctions did not result in increased binding of annexin V to the cell surface. This indicates that loss of cell polarity did not result in the exposure of PS at the cell surface, although the treatment did result in increased COM attachment (Fig. 3). As can be seen in Figure 2B, the calcium ionophore A23187 treatment that is known to result in loss of membrane lipid asymmetry [31] resulted in the binding of annexin V to IMCD cells, which confirms the surface exposure of PS on the cells. It should be noted that not all of the cells respond to the increase in internal calcium at the same dose or time of exposure. With the use of a higher concentration or longer exposure time of ionophore, all cells would eventually expose PS on their surfaces. We used this concentration and time of exposure attempting to minimize the severe toxic effects on the cells so as to avoid disruption of the monolayer. We have previously shown that A23187 exposure of IMCD cells at the concentration and time of exposure used in this study enhanced COM attachment [12]. In this study, we confirmed the speculated exposure of PS on the surface following A23187 treatment (Fig. 2B).

To confirm further the role of PS exposure on the cell surface in COM crystal attachment to the IMCD cells exposed to EGTA, we performed crystal attachment inhibition studies using unlabeled annexin V to block the PS binding site on the cell surface. Figure 3 shows the enhanced COM crystal attachment induced by the loss of cell polarity. Nearly twice the amount of COM attached to the IMCD cells following the loss of cell polarity induced by EGTA treatment, paralleling results we have obtained previously [19]. It also can be seen that the addition of annexin V before crystal attachment to IMCD cells that have been treated with EGTA had very little effect on COM attachment. It is apparent that a loss of IMCD cell polarity does not result in surface exposure of PS and that the attachment of COM crystals to the cells must be by a different mechanism.

DISCUSSION

This study was conducted to investigate whether PS exposure on the cell membrane surface plays a role in COM crystal attachment to IMCD cells that have lost their polarity as well as to cells that have lost their plasma

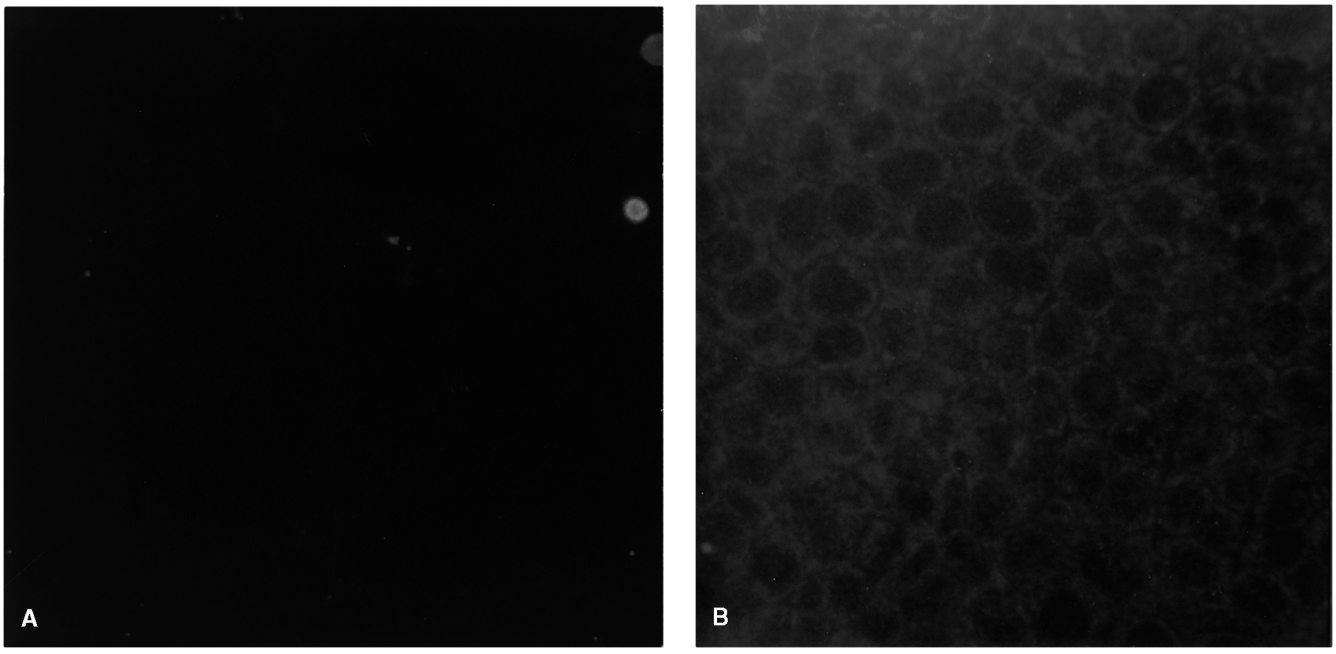

Fig. 1. FITC-annexin V binding to cell membranes following enrichment with exogenous phosphatidylserine (PS). This serves as a positive control of annexin V binding. Photomicrographs of fluorescently labeled (FITC) annexin V binding to inner medullary collecting duct (IMCD) cells in culture are shown. (*A*) Control cells with no PS enrichment. (*B*) Cells enriched with exogenous PS. Original magnification ×200.

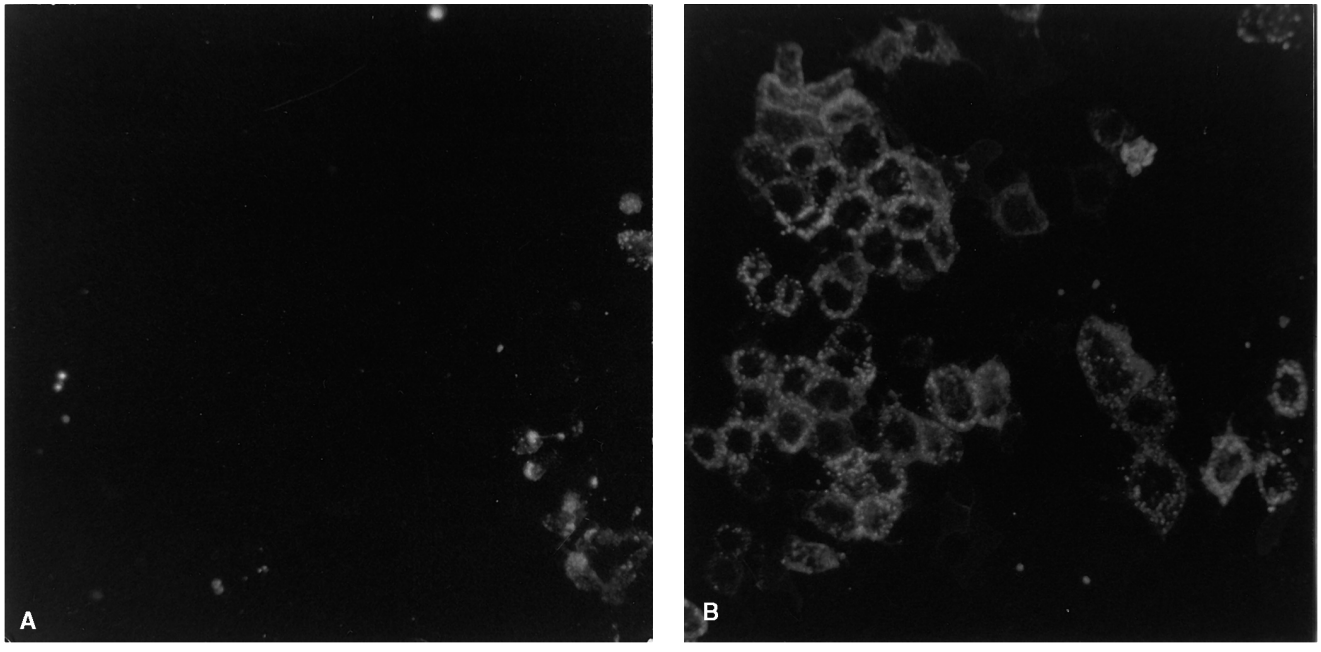

Fig. 2. FITC-annexin V binding to cell membranes of IMCD cells following two different modes of injury. (*A*) Cells that have lost their polarity by treatment with 3 mmol/L EGTA for 15 minutes at 37°C. (*B*) Cells that have lost their membrane lipid asymmetry by treatment with 5 μmol/L calcium ionophore A23187 for 30 minutes at 37°C. Original magnification ×200.

membrane lipid asymmetry following cell or tissue injury. We have shown that the increase in crystal attachment following cell or tissue injury was due to the exposure of molecules on the cell surface that are not normally accessible to crystals. In addition, we have shown that more than one mechanism may be involved in the increased attachment of crystals to renal cells that have been injured.

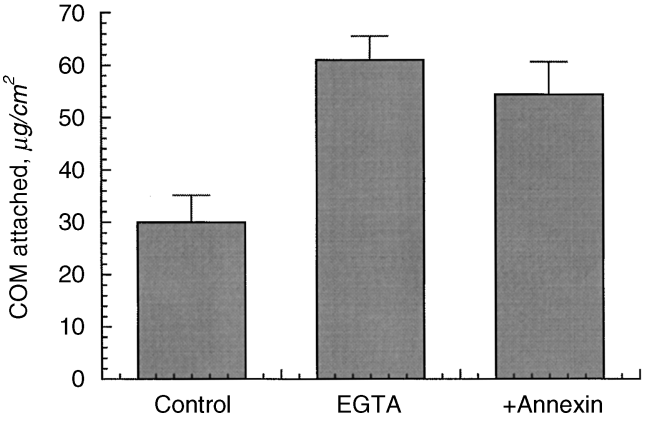

Fig. 3. Annexin V inhibition of crystal attachment to EGTA-treated IMCD cells. The control is untreated cells. The column labeled EGTA represents cells that have lost their polarity by treatment with 3 mmol/L EGTA for 15 minutes at 37°C. (Significant difference from control cells $P < 0.01$, unpaired Student t test). The column labeled +Annexin represents calcium oxalate monohydrate (COM) attachment to EGTA-treated cells that have been incubated with annexin V prior to crystal attachment. Five μmol/L of unlabeled annexin V were added to the EGTA-treated cells and incubated for 15 minutes at 37°C before COM attachment. (Significant difference from EGTA, $P = 0.09$, unpaired Student t test). Results are expressed as the mean of COM attached (μg/cm^2) of cell surface. Error bars indicate standard error of the mean. ($N = 5$)

Previously, we speculated that the increase in COM attachment to IMCD cells in culture that had lost their polarity following tight junction breakdown was due to either exposure of basement membrane components or the rearrangement of basolateral membrane components to the cell surface [19]. The exposure of these previously inaccessible membrane components on the membrane surface following loss of cell polarity clearly facilitates crystal attachment. The same study, using a monoclonal antibody to a basolateral component, showed that basolateral components were rearranged to the apical membrane surface upon the breakdown of tight junctions with EGTA treatment [19]. A subsequent study on the effect of phospholipid composition of IMCD cell membranes on crystal attachment to the membranes [10] and a study in which the cells were injured with calcium ionophore A23187 to induce loss of membrane lipid asymmetry [12] led us to speculate that PS exposure on the cell surface may be responsible for increased crystal attachment following this form of cell injury.

Figure 1 shows that when IMCD cell membranes are enriched with PS, the surface of the cells exhibits extensive binding of FITC-annexin V. The enrichment of membranes with PS was used as a positive control of annexin V binding to PS in this study. In a previous study, we have found that injury of IMCD cells with calcium ionophore A23187 to induce the loss of membrane lipid asymmetry enhanced the attachment of COM crystals to the cells [12]. The same study showed that annexin V inhibits COM attachment under these conditions, indicating that COM attaches to membrane PS, although we did not directly confirm that PS was actually on the cell surface. This was confirmed in the present study (Fig. 3), which shows that when IMCD cells were injured with A23187, the number of cells that bind annexin V increased, indicating that PS is exposed on the cell luminal surface. This observation is not only consistent with literature indicating that treatment with A23187 results in the exposure of anionic phospholipids that are normally confined to the inner leaflet of the plasma membrane [35], but also firmly establishes the role of PS in crystal attachment. In a recent study, it has also been shown that oxalate induces the exposure of PS on the surface of IMCD cells in culture, and the exposure of PS was temporal to the attachment of COM crystals to the cells [31].

Disruption of the IMCD cell tight junctions with EGTA to induce the loss of cell polarity (Fig. 2A) resulted in little increase in the binding of FITC-annexin V to the cells, indicating that loss of cell polarity did not result in PS exposure at the cell surface. In addition, when we tried to inhibit COM attachment to the EGTA-treated cells (Fig. 3) with unlabeled annexin V, there was no decrease in crystal attachment. Clearly, COM crystals are not attaching to PS in this instance, but probably to basement membrane or non-PS basolateral membrane components that become exposed when tight junctions are disrupted. The multitude of basolateral, basement membrane, and junctional components, as well as a large cast of cytokines and other molecules secreted by injured cells makes it extremely difficult to speculate on the exact molecules involved in enhanced crystal attachment as a result of loss of cell polarity. However, this mechanism in no way precludes any of the many molecules proposed in the literature from being involved in crystal attachment or nucleation. This is an important area of investigation being considered by many investigators [9–31, 36–38].

Not all people with crystalluria form stones [39]. Even patients with the same metabolic disorder do not all form stones [40]. This indicates that the luminal surface of kidney epithelial cells do not normally express crystal attachment molecular arrays to accommodate crystal attachment for a time period sufficient for clinically significant stone development. We suggest that normal IMCD cells do not attach stone crystals, but that they transform into cells that can attach crystals following cell injury. This injury could involve a small area of tissue or injury to a few cells while not involving the entire luminal surface. This could explain why massive numbers of kidney stones are not formed during each episode or large amounts of cells or proteins are not seen in the urine that would indicate extensive renal injury.

Our results show that there are at least two distinct mechanisms of crystal attachment to epithelial cells fol-

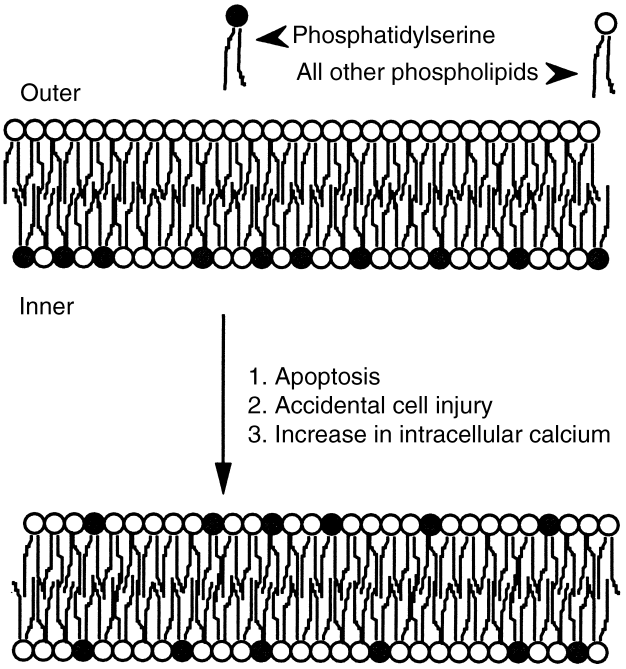

Fig. 4. Schematic representation of loss of membrane phospholipid asymmetry following cell injury.

lowing injury. First, there are crystal–PS interactions that could occur as a result of exposure of PS on the cell surface of cells that have lost their membrane lipid asymmetry, as illustrated in Figure 4. This may be a result of apoptosis or cell injury. Apoptosis can be differentiated from other forms of cell death in that it requires suicide mechanisms and is energy dependent [41, 42], while nonapoptotic cell injury could be caused by physical stimuli such as crystals, toxic urinary conditions resulting from ionic challenges, metabolic disorders, or pharmaceutically induced injury. The rate of apoptosis in the kidney is thought to be quite high because of constant remodeling of the kidney nephron [42]. This process is normally tightly controlled, but could become abnormal in response to ischemic or toxic insult to the kidney tubule. The second mechanism is based on crystal interaction with basolateral or basement membrane components that could be exposed as a result of the loss of cell polarity, as illustrated in Figure 5. This type of injury may allow cell membrane components that are usually sequestered to the basolateral surface or in the tight junction region to migrate to the apical surface of the cell. Epithelial cell polarization plays a critical role in maintaining a cell's vectorial functions, including movement of water, ions, and macromolecules. The loss of epithelial cell polarity is seen in several disease states in association with tissue injury, ischemia in kidney tubules, microvillus inclusion disease, and polycystic kidney disease [43, 44]. Dividing cells during epithelial wound repair do not become polarized until their tight junctions are established and the apical and basolateral domains are created [44]. During the repair of epithelial wounds, the loss of cell polarity is seen not only in the wound area, but also in uninjured cells surrounding the area of trauma [45, 46]. There are several examples of bacteria and viruses that cannot enter the apical surface of polarized epithelial cells but are able to attach and enter from the basolateral surfaces [47–49]. When these cells lose their polarity, either by experimental manipulation or tissue injury, the infectious agent can then enter the cell anywhere on the cell surface. Just as we have proposed that the attachment of crystals to cells is related to the loss of their polarity in urolithiasis, the authors of the bacterial and viral studies speculated that molecules that facilitate the binding of these infectious agents to the cells are normally sequestered on the basolateral surface of the cells and are not normally accessible.

The mechanism of crystal interaction to the luminal surface of IMCD cells following these two different types of injury will undoubtedly be different. The exposure of PS on the luminal surface of IMCD cells resulting from the A23187 treatment provides a specific calcium binding molecule on the cell surface that could form a molecular contact with the calcium atom in the surface atomic structure of calcium oxalate crystals [6]. The strength of PS-calcium oxalate crystal molecular contact during attachment would be significantly increased if a number of PS molecules were to group together on the cell surface. PS-rich regions on the cell surface could also serve as calcium-containing crystal nucleation sites. Studies have shown that during calcium oxalate crystal nucleation in the presence of acidic phospholipids that an insoluble Ca-PS-Pi complex formed [50].

The injury induced by EGTA treatment results in the loss of cell polarity and the presentation on the cell luminal surface molecules normally sequestered on the basolateral surface, the tight junction, or even on the basement membrane. These molecules usually demonstrate cell–cell and cell–basement membrane attachment properties and would naturally be "crystal sticky." The EGTA treatment therefore leads to the exposure of a broad spectrum of molecules on the cell's luminal surface, thereby making the cell an easy attachment target for crystals as well as many other components of stone former urine such as macromolecules, cellular debris, and a myriad of urinary ions.

It is very likely that at sites of tubule epithelial injury both of these mechanisms may play a role in crystal attachment or nucleation. The understanding of these two mechanisms of crystal attachment that appear to be related to the magnitude and type of tissue or cell injury is necessary before any reasonable pharmacological intervention can be designed.

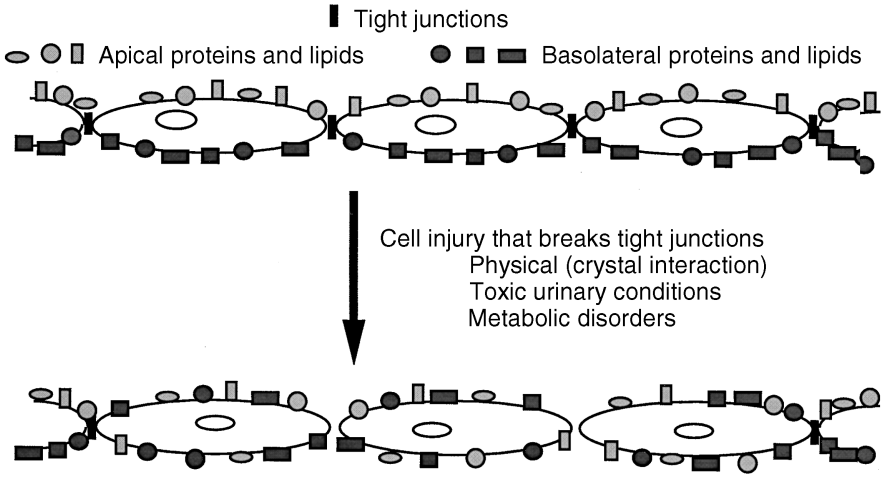

Fig. 5. Schematic representation of loss of cell polarity following cell or tissue injury.

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